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14. ABSTRACT Breast cancer stem cells exist in several breast cancer cell lines based on recognized surface marker CD44+/CD24–or low or Hoechst efflux/ABCG2. The increased radiation resistance of breast cancer stem-like cells (CD44+/CD24–or low) was found in MCF-7, HCC1937, and MDA-MB-231 cells lines. The differential radiation resistance among the subpopulation might not rely on the NHEJ activity. Differential activation of ATM pathway could contribute to differential radiation resistance among the subpopulations of breast cancer cell lines. However, the inhibition of ATM activation blocked the growth more significantly in non stem-like cells than in stem-like cells after radiation.

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CD44/CD24, Cancer stem-like cells, radiation sensitivity, non-homologous end joining DNA repair, response to DNA damage

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Introduction

The existence of cancer stem cells is a hypothesis put forth both to explain the initiation of cancer and the recurrence of cancer after treatment. Evidence supporting the presence of cancer stem cells has been developed for in hematologic malignancies and solid tumors. In breast cancer, $CD44^{+}/CD24^{-/low}$ tumorigenic stem cells have been identified with as few as 100 cells of these cells being able to form tumors in mice [1, 2]. These cells exhibit unlimited propagation and could give rise to subpopulations of tumorigenic and non-tumorigenic cells. These CD44 positive cells also expressed many known stem cell markers and the presence of $CD44^{+}/CD24^{-/low}$ cells correlated with the survival of breast cancer patients [3]. In addition to breast cancer tissue, cancer stem cell-like cells have also been found in carcinoma-derived cell lines [4, 5] . The ability to establish various breast cell lines has already indicated that within the cancers there are cells with clonegenic ability and which, in some circumstances, possess the characteristics of cancer stem cells. For example, several breast cancer cell lines contain a subpopulation of $CD44^{+}$ $/CD24^{-/low-\ positive}$ with epithelial specific antigens or a side population with stem cell characteristics such as Hoechst efflux and sphere formation. These cells are capable of self–renewal and have the ability to give rise to differentiated cell populations. However, the responses of the breast cancer stem-like cells to chemotherapy

and radiotherapy have not been systematically investigated. The understanding of the characteristics of response of these types of cells to chemotherapy and radiotherapy could be essential for development of different treatments and potentially cure of breast cancer. In this project, our attempt is to isolate cancer stem cell-like cell populations from cultured breast cancer cell lines to identify the differential molecular responses breasta cancer stem cell-like populations to radiation and explore new molecular targets for improvement of the radiation therapy. Recently, two publications have addressed the response of breast cancer stem-like cells based on human breast cancer cells line and mouse mammary cells suggesting the resistance of breast cancer stem–like cell and mammary progenitor cells to radiation [6, 7].

Body

I. Characterization of subpopulations in established breast cancer cell lines.

In order to demonstrate the existence of subpopulations in breast cancer cell lines, we examined several breast cancer cell lines with the Hoechst 33342 efflux/ABCG2 and CD44/CD24 surface marker analysis in six breast cancer cell lines (Figure 1). The side population exhibiting efflux of Hoechst 33342 is shown in Figure 1 with the percentage of cells exhibiting dye efflux being 1.3% in MCF-7 cells, 0.6% in MDA-MB-436 cells, 0.4% in MDA-MB-231 cells, and 0.1% in HCC1937 cells. Neither. MDA-MB-453 nor HCC38 showed a Hoechst 33342 efflux side population (Figure1). It has been mentioned the Hoechst 33342 efflux is predominately controlled by ABCG2 transporters in mammalian cell membrane. To verify the relationship between ABCG2 and Hoechst 33342 efflux in breast cancer cell lines, we next examined the expression of ABCG2 in cell lines with positive Hoechst 33342 efflux (Figure 2). As shown a small portion of ABCG2 positive cells could be found in some of the cell lines with positive Hoechst efflux, such as MCF-7 (0.7%) and HCC1937 (0.4%) while MDA-MB-231 cells, though exhibiting a side population of positive Hoechst efflux, had no ABCG2 positive cells, suggesting that ABCG2 responsibility for Hoechst 33342 efflux may be cell line specific. In addition to Hoechst 33342/ABCG2 analysis, we conducted the

analysis of breast cancer cell lines by CD44 and CD24 expression which are well recognized markers for breast cancer stem-like cells. Depending on specific cell lines, the portion of subpopulation with $CD44^+/CD24^-$ ranged from 0 to 97%. (Figure 3A). Two mesenchyme-like breast cancer cell lines MDA-MB-436 and MDA-MB-231 showed the highest portion of a $CD44^+/CD24^-$ subpopulation, 97% and 55.3% respectively. For other epithelium-like breast cancer cell lines, the $CD44^+/CD24^-$ subpopulation varied from 0 to 5.9 %. One non-tumorigenic epithelial cell line, MCF10A, also showed that 3.1 % of the cells were $CD44^+/CD24^-$ positive. Hence, it seems that mesenchyme-like breast cancer cell lines contain higher percentages of $CD44^+/CD24^-$ cells than epithelial cancer cell lines and that the $CD44^+/CD24^-$ subpopulation may be present in both tumogenic and non-malignant breast cells. Further analysis of epithelial specific antigen (ESA) to identify the origin of the cell lines demonstrated that in MCF-7 and HCC1937 cells 98% and 93% of cells respectively were ESA positive while in MDA-MB231 cells only 65% cells were ESA positive indicating that epithelium to mesenchymal transition could accompany the increased portion of $CD44^+/CD24^-$ cells (Figure 3B) .

Does the subpopulation of cells always maintain its $CD44^+/CD24^-$ surface markers or do the surface markers vary/switch with time? To answer this question we analyzed the CD44/CD24 surface marker in presorted MCF-7, MDA-MB-231, and HCC1937 cells. After sorting into $CD44^+/CD24^{-or\ low}$, $CD44^+/CD24^+$ (MDA-MB-231), $CD44^-/CD24^{high}$ (MCF-7 and HCC1937) populations, cells were cultured for 6 days or two weeks and were then reanalyzed for expression of CD44/ CD24 status. Interestingly, 70 to 80% of cells maintained the original CD44/CD24 phenotype for 6 days after sorting. However, after two weeks of culture the following transitions were observe: $CD44^+/CD24^{-or\ low}$ could change to $CD44^-/CD24^{high}$ (MCF-7 and HCC1937) or $CD44^+/CD24^+$ (MDA-MB-231) and $CD44^-/CD24^{high}$ (MCF-7 and HCC1937) or $CD44^+/CD24^+$ (MDA-MB-231) cells could develop to $CD44^+/CD24^{-or\ low}$ cells (Figure 4) . In fact,

altered expression of the surface markers CD44/CD24 occurred even in same breast cell line at different passages. For example, with the MCF-7 cell line the percentage of CD44 positive cells increased with increasing cell passage. In HCC1937 cell line, an obvious subpopulation of $CD44^{+}/CD24^{-}$ could be found after 15-17 passages with the first culture after obtaining cells from ATCC as passage 1. At passage 21, 98% of cells had become $CD44^{+}/CD24^{-}$ (Figure 5, top panels). This altered expression of surface markers was not limited to CD44 and CD24. The expression of ABCG2 also changed with cell passage number (Figure 5, bottom panel). Interestingly, the percentage of ABCG2 positive cells decreased in high passages of HCC1937 even though 98% cells were of the $CD44^{+}/CD24^{-}$ phenotype. The observation suggests that there may not be a close relationship between the $CD44^{+}/CD24^{-\ or\ low}$ phenotype and ABCG2 positive /Hoechst efflux phenotype.

2. Post-radiation survival analysis of $CD44^{+}/CD24^{-or\ low}$ (MCF-7, HCC1937, and MDA-MB231) vs $CD44^{-}/CD24^{high}$(MCF-7 and HCC1937) or $CD44^{+}/CD24^{+}$ (MDA-MB-231)

According to the original plan, we first attempted to sort the side population by Hoechst 33342 efflux and then further identify these cells by the expression of CD44 and CD24. Detection of Hoechst 33342 requires an ultraviolet laser and unfortunately neither of the two sorters available in our facility, the FACSVantage and the FACSAria SE, are currently equipped ultraviolet laser. Fortunately, the appropriate lasers have been installed so that it is now feasible to sort by Hoechst 33342 efflux. As an alternative, we sorted breast cancer cell lines directly based on the presence of the surface markers CD44 and CD24 which are currently well recognized as markers of stem-like or tumor initiating cells in breast cancer cells. Based on the plating efficiency, the percentage of $CD44^{+}/CD24^{-or\ low}$ cells, and the universality of use of the cell line, we selected three breast cancer cell lines, MCF-7, MDA-MB-231, and HCC1937 (passage 6-17) to sort by their surface marker expression of CD44 and CD24. Sorted cells were cultured for 3 or 5 days and then radiated.

Clonogenic survival analysis of the sorted cells is shown in Figure 6. In MCF-7 and HCC1937 cells increased radiation resistance was seen in the $CD44^{+}/CD24^{low}$ subpopulation compared to $CD44^{-}/CD24^{high}$ and non-sorted cells populations at all radiation doses (Figure 6, left and right panel). Further analysis of the survival fraction at 2Gy, which approximates clinical doses, showed that the survival fraction in $CD44^{+}/CD24^{low}$ subpopulation of MCF-7 and HCC1937 were increased by over 50 % compared to the $CD44^{-}/CD24^{high}$ subpopulation (data was shown in Figure 6 of 2007 annual report)). In contrast to the previous finding in 2007 annual report, MDA-MB-231 cells showed similar results to MCF7 and HCC1937 cells with the $CD44^{+}/CD24^{-}$ subpopulation being more radio-resistant than $CD44^{+}/CD24^{+}$ subpopulation. These experiments have now been repeated four times with similar results being obtained. The middle panel in Figure 6 represents one of the four experiments. The change in results from the annual report results from a switch in the source of DMEM medium from Mediatech Inc to Invitrogen Corp. The medium from Invitrogen greatly improved the efficiency of colony formation of MDA-MB-231 cells. In addition, we examined apoptosis after radiation by analysis of cleaved PARP products. We did not find of PARP cleavage products in cells 6 hour after radiation at 10 to 12 Gy. In contrast, cells exposed to UV for 6 hour showed significant cleavage of PARP in western blot analysis, indicating a potential difference in cell response to gamma and UV radiation.

To identify further the relationship $CD44^{+}/CD24^{-or\ low}$ phenotype and radiation resistance, we conducted clonogenic survival analysis with MCF-7 cells in culture for 2 weeks after sorting to see if altered CD44 and CD24 phenotype which occurs after longer culture after sorting affects the radiation resistance of cells. The expression of CD44 and CD24 in the $CD44^{+}/CD24^{-or\ low}$ and $CD44^{+}/CD24^{+}$ subpopulation before and after culture is shown in Figure 4. After culture for 2 weeks after sorting the cells received 2 Gy radiation. The clonogenic survival fractions are shown in Figure 7 and there was no significant difference between the populations assayed prior to and after culture ($p>0.15$, Student T-test) suggesting that the $CD44^{+}/CD24^{-or\ low}$

phenotype is determinant for radiation resistance. On the other hand, the CD44/CD24 phenotype of non-sorted MCF-7, HCC1937, and MDA-MB-231 cells did not change, i.e. there was no increase of a $CD44^{+}/CD24^{-\text{or low}}$ subpopulation, followed for 10 days after radiation.

3. Examine the stem cell marker expression in $CD44^{+}/CD24^{-\text{or low}}$ vs. $CD44^{-}/CD24^{\text{high}}$ (MCF-7 and HCC1937) or $CD44^{+}/CD24^{+}$ (MDA-MB-231)

Does the $CD44^{+}/CD24^{-\text{or low}}$ phenotype in breast cancer cells represent true stem-like cells? To address this issue, we preliminarily examined the expression of genes related to myeloid stem cells and embryonic stem cells. In MCF-7 cells Notch 1 expression in the $CD44^{+}/CD24^{\text{low}}$ subpopulation was about 30% higher than in the $CD44^{-}/CD24^{\text{high}}$ subpopulations (Figure 8). Similar analyses are being undertaken for other genes with increased expression in myeloid and embryonic stem cells.

4. Analysis of end-joining of double-strand DNA breaks in $CD44^{+}/CD24^{-\text{or low}}$ vs. $CD44^{-}/CD24^{\text{high}}$ (MCF-7 and HCC1937) or $CD44^{+}/CD24^{+}$ (MDA-MB-231) subpopulations.

In order to identify differential expressed proteins in the non-homologous end joining (NHEJ) complex in stem-like breast cancer cells, NHEJ activity was first examined in vivo in $CD44^{+}/CD24^{-\text{or low}}$ and $CD44^{-}/CD24^{\text{high}}$ (MCF-7 and HCC1937) or $CD44^{+}/CD24^{+}$ (MDA-MB-231) subpopulations. A plasmid with SV-40 promoter driven luciferase reporter gene, pGL2-Control was linearized by Stu1 to create blunt ends between the promoter and the luciferase reporter gene. The Stu1 digested plasmid was examined by agarose electrophoresis to verify the linearization. Two days after sorting cells were transfected with linearized reporter plasmid following radiation. The luciferase activity was measured and standardized by expression of co-transfected β-galactosidase. As shown in Figure 9, no increase *in vivo* NHEJ was seen in cells with in $CD44^{+}/CD24^{-\text{or low}}$ phenotype. In contrast, increased *in vivo* NHEJ was found in $CD44^{-}/CD24^{\text{high}}$

subpopulation of HCC1937 and $CD44^+/CD24^+$ subpopulation of MDA-MB-231 cells. Combined with post-radiation clonogenic survival results, the analysis of *in vivo* NHEJ suggests that differential clonogenic survival between $CD44^+/CD24^{-\text{or low}}$ sub population and $CD44^-/CD24^{\text{high}}$ subpopulation may not be a simple function of NHEJ, at least, for the acute response phase in MCF-7 and HCC1937 cells after radiation. Only MDA-MB-231 cells showed a slightly increased *in vivo* NHEJ in the $CD44^+/CD24^+$ subpopulation. To explore further the NHEJ function in MDA-MB-231 cells, we conducted an *in vivo* analysis of NHEJ activity in subpopulations (Figure 10). At radiation doses of 6 and 8 Gy, there were no significant difference in DNA ligation activity between $CD44^+/CD24^-$ and $CD44^+/CD24^+$ subpopulations.

5. DNA damage-repair response after radiation in $CD44^+/CD24^{-\text{or low}}$ vs. $CD44^-/CD24^{\text{high}}$ (MCF-7 and HCC1937) or $CD44^+/CD24^+$ (MDA-MB-231) subpopulation of three cell lines.

To investigate the potential differences in NHEJ activity of $CD44^+/CD24^{-\text{or low}}$ vs. $CD44^-/CD24^{\text{high}}$ (MCF-7 and HCC1937) or $CD44^+/CD24^+$ (MDA-MB-231) subpopulations, we examined the expression of components of the NHEJ complex by western blot analysis (Figure 11A). The results showed that there were no significant differences in expression Ku 80, Ku70, and PARP-1 between the two subpopulations in the HCC1937 and MDA-MB-231 cell lines. Expression of these components will also be examined in subpopulations of MCF-7 cells. DNA-PK showed a one-fold decreased expression in the $CD44^+/CD24^{-\text{or low}}$ subpopulation in both cell lines after radiation. Examination of expression of DNA ligase IV has been unsuccessful because of the poor quality of antibody available and the investigation of phosphorylation of DNA-PK is limited due to the availability of both nuclear protein and antibodies of adequate quality and specificity.

Because our preliminary data could not completely support the assumption that increased NHEJ is responsible to increased radiation resistance in $CD44^+/CD24^{-\text{or low}}$ subpopulation, we next examined the

activation of ATM/ATR pathway which is important for DNA damage/repair responses (Figure 11B-E). One hour after radiation the phosphorylation of histone 2AX (γ-H2AX) was reduced by about one fold in $CD44^{+}/CD24^{low}$ MCF-7 cells compared to $CD44^{-}/CD24^{high}$ MCF-7 cells. However, a difference of phosphorylation of H2AX in MCF-7 was not found in MDA-MB231 and HCC1937 cells. (Figure 11B, upper panel). However, increased phosphorylation of ATM was found in the $CD44^{+}/CD24^{-or\ low}$ subpopulation of both MCF-7 and MDA-MB-231 cells (Figure 11C). To verify the increased activation of ATM pathway in the $CD44^{+}/CD24^{-}$ subpopulation, we next examined the phosphorylation of downstream targets of ATM and found that both BRCA-1 and p53 showed increased phosphorylation in the $CD44^{+}/CD24^{-}$ subpopulation of MDA-MB-231 cells (Figure 11D). The phosphorylation of ATM did not differ between the $CD44^{+}/CD24^{-}$ and $CD44^{+}/CD24^{+}$subpopulations of MDA-MB-231 cells after radiation in three individual experiments (Figure 11E). The differences seen in the previous annual report may have been caused by a variety of technical problems which have now been resolved. Similarly, the differences in phosphorylation of ATM previously found in the HCC1937 and MCF7 cell lines could not be demonstrated in repeat experiments. Taken together the preliminary results presented now suggest that the ATM pathway could contribute to the differential radiation response between stem-like and non stem breast cancer cells.

6. Co-immunoprecipitation detection of NHEJ associated proteins. To explore the role of NHEJ associated proteins in conferring radiation resistance to stem cells, we conducted co-immunopreciopitation with nuclear extract from the $CD44^{+}/CD24^{-}$ and $CD44^{+}/CD24^{+}$ subpopulations of MDA-MB-231 cells with antibodies against DNA-PK, phosphorylated-ATM, Ku70, and Ku80 and then analyzing the co-precipitated proteins by separation on 4-15% SDS-PAGE and followed by staining with GelCode (Invitrogen) (Figure 12). As shown there was no significant difference in the pattern of precipitated proteins. To identify the individual proteins co-precipitated, we collected each stained protein band in the gel and tried to identify the

proteins by Matrix-Assisted Laser Desorption Ionization – Time-Of-Flight (MALDI-TOF). To date this analysis has not been sucessful. However, we were able to demonstrate consistently that ATM was associated with PAPR, Ku70, and Ku80, all components of NHEJ (Figure 13), but that there was no differences seen between the $CD44^+/CD24^-$ and $CD44^+/CD24^+$ subpopulations.

7. **ATM inhibitor decreased the growth of $CD44^+/CD24^+$ subpopulation**. In view of the increased phosphorylation of ATM in the $CD44^+/CD24^-$ subpopulation after radiation, we tested the hypothesis that the inhibition of phosphorylation of ATM would increase the radiosensitivity of the $CD44^+/CD24^-$ subpopulation. Post-sorted MDA-MB-231cells were treated with 2-Morpholin-4-yl-6-thianthren-1-yl-pyran-4-one, an ATM kinase inhibitor for 24 hours at 5 and 20 nM beginning 2 hours before the radiation. The proliferation assay showed that the $CD44^+/CD24^+$ subpopulation was more sensitive to ATM kinase inhibition than the $CD44^+/CD24^-$subpopulation (Figure 14). These results suggest that the activation of ATM would be important for cell survival or recovery post radiation.

Key Research Accomplishments:

In the two years of research on this project, we have characterized the cancer-stem cell subpopulation in breast cancer cell lines by cells surface marker and Hoechst efflux and successfully sorted $CD44^+/CD24^{-\text{or low}}$ (MDA-MB-231, MCF-7, HCC1937) and $CD44^-/CD24^{high}$ (MCF-7 and HCC1937) or $CD44^+/CD24^+$ (MDA-MB-231) subpopulations. We have examined the expression of Notch gene, one of markers of the breast stem cells in the sorted subpopulations. We have examined clonogenic survival of $CD44^+/CD24^{-\text{or low}}$ (MDA-MB-231, MCF-7, HCC1937) and $CD44^-/CD24^{high}$ (MCF-7 and HCC1937) or $CD44^+/CD24^+$ (MDA-MB-231) subpopulations and apoptosis after radiation. We investigated the NHEJ activity *in vivo* and *in vitro* before and after radiation and expression of NHEJ component proteins among the subpopulations. NHEJ does not appear to account for the radiation resistance of stem cell like cells in breast cancer cell

lines. We explored the activation of ATM pathway by radiation among the subpopulations, identified that ATM was an associated protein of PARP, Ku70, and Ku80, and showed that the ATM kinase inhibitor blocked the growth of non-stem cells other than that of stem-like cells after radiation.

Reportable Outcomes

Conference presentation

1. **Hong Yin**, Jonathan Glass. Characterization of the radioresistance of stem-cell like cells in breast cancer cell lines (#4597)AACR 2008 Annual Meeting, April 12-16, San Diego CA.

2. N. S. Luraguiz, T. Yong, **H. Yin**, A. Sun Targeting notch signaling and estrogen receptor pathways in human breast cancer stem cells. ASCO 2008 Annual Meeting J Clin Oncol 26: 2008 (May 20 suppl; abstr 22066)

Conclusions

Breast cancer stem cells exist in several breast cancer cell lines based on identification by the recognized surface markers $CD44^{+}/CD24^{-or\ low}$ or by Hoechst efflux/ABCG2. The increased radiation resistance of breast cancer stem-like cells ($CD44^{+}/CD24^{-or\ low}$) was found in MCF-7, HCC1937, and MDA-MB-231 cells lines. The differential radiation resistance among the subpopulation might not result on the NHEJ activity. Differential activation of ATM pathway could contribute to differential radiation resistance among the subpopulations of breast cancer cell lines. However, the inhibition of ATM activation blocked the growth more significantly in non stem-like cells than in stem-like cells after radiation.

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Appendices

I.

Abstract Number:	4597
Session Title:	Therapeutic and Radiation Responsiveness of Stem Cells
Presentation Title:	Characterization of the radioresistance of stem-cell like cells in breast cancer cell lines
Presentation Start/End Time:	Tuesday, Apr 15, 2008, 1:00 PM - 5:00 PM
Location:	Exhibit Hall B-F, San Diego Convention Center
Poster Section:	16
Poster Board Number:	4
Author Block:	Hong Yin, Jonathan Glass. Louisiana State Univ. Health Sciences Ctr., Shreveport, LA

The presence of cancer stem cells may explain cancer initiation and recurrence after treatment. To demonstrate stem cell-like cell (SCLC) subpopulations in breast cancer cell lines, we examined by flow cytometry Hoechst 33342 efflux, ABCG2 protein, and CD44/CD24 surface markers. Hoechst 33342 efflux identified stem cells in MCF-7 (1.3%), MDA-MB-436 (0.6%), MDA-MB-231 (0.4%), and HCC1937 (0.1%) but none in MDA-MB-453 and HCC38 cells. The presence of ABCG2 correlated with Hoechst 33342 efflux in MCF-7 and HCC1937 but not

MDA-MB-231 cells, suggesting that ABCG2 activity responsible for Hoechst 33342 efflux may be cell line specific. Analysis of CD44 and CD24 expression, known markers of SCLC, showed a subpopulation of $CD44^{+}/CD24^{-}$ cells ranging from 97% and 55.3% positive cells in mesenchymal-like breast cancer cell lines MDA-MB-436 and MDA-MB-231 to 0% to 5.9 % in epithelium-like cell lines, MCF-7 and HCC1937. To determine if the $CD44^{+}/CD24^{-}$ population varies with time, the CD44/CD24 surface markers were assayed at 6 days and 2 weeks of culture after sorting. The $CD44^{+}/CD24^{-or\ low}$ (MCF-7, MDA-MB-231, and HCC1937 cells), $CD44^{+}/CD24^{+}$ (MDA-MB-231), $CD44^{-}/CD24^{high}$ (MCF-7 and HCC1937) populations were reanalyzed after 6 days of culture with 70-80% of cells maintaining the original CD44/CD24 phenotype. After two weeks of culture the following transitions occurred: 1) $CD44^{+}/CD24^{-or\ low}$ cells changed to $CD44^{-}/CD24^{high}$ cells (MCF-7 and HCC1937) or to $CD44^{+}/CD24^{+}$ (MDA-MB-231); and 2) $CD44^{-}/CD24^{high}$ (MCF-7 and HCC1937) and $CD44^{+}/CD24^{+}$ (MDA-MB-231) cells became $CD44^{+}/CD24^{-or\ low}$ cells. As SCLC are radiation resistant we examined the radiation sensitivity of $CD44^{+}/CD24^{-or\ low}$ cells with a clonogenic assay on sorted populations of MCF-7, MDA-MB-231, and HCC1937 cells. Increased radiation resistance was seen in the $CD44^{+}/CD24^{-or\ low}$ subpopulations compared to $CD44^{-}/CD24^{high}$ (MCF-7 and HCC1937), $CD44^{+}/CD24^{+}$ (MDA-MB-231) cells, and non-sorted cells. Analysis of the survival fraction at 2Gy showed the survival fraction in $CD44^{+}/CD24^{low}$ subpopulation of all three cell lines increased by > 40 % compared to the $CD44^{-}/CD24^{high}$ (MCF-7 and HCC1937) and $CD44^{+}/CD24^{+}$ (MDA-MB-231) cells. Increased radiation resistance was not a function of non-homologous end joining (NHEJ) as there was no in vivo change in NHEJ activity in $CD44+/CD24^{-\ or\ low}$ cells nor altered expression of the NHEJ related proteins Ku 80, Ku70, PARP-1, and DNA-PK. Differences were found in the activity of ATM pathway between $CD44^{+}/CD24^{-or\ low}$ and the $CD44^{-}/CD24^{high}$ (MCF-7 and HCC1937) or $CD44^{+}/CD24^{+}$ (MDA-MB-231) subpopulations suggesting that radiation resistance of SCLC might result from altered ATM activity. These studies give credence to the hypothesis for SCLC in breast cancer cell lines, that properties of these populations vary between cell lines, and that radiation resistance of SCLC may be from altered ATM pathway.

2.

Abstract No:	22066
Citation:	*J Clin Oncol* 26: 2008 (May 20 suppl; abstr 22066)
Author(s):	N. S. Luraguiz, T. Yong, H. Yin, A. Sun
Abstract:	**Background:** Breast cancer stem cells have been identified as ESA+CD44+CD24-Lin- cells, which may account for disease relapse and metastasis. Deregulation in stem cell self-renewal pathways such as Notch, Wnt and Hedgehog signaling have been implicated in mammary transformation. In humans, high levels of Notch1 are associated with reduced patient survival. Inhibition of Notch signaling has been proposed as a potential strategy in treating breast cancer. Tamoxifen, a modulator of estrogen receptor (ER), is currently used for the treatment of both early and advanced ER+ breast cancer. It is unclear whether ER+ breast cancers

originate from ER+ or ER- mammary stem/progenitor cells. Here, we investigate the molecular mechanisms of Notch signaling and ER interaction in breast cancer stem cells, and evaluate Notch inhibition and estrogen antagonist in targeting breast cancer stem cells.

Methods: CD44+CD24- breast cancer stem cells are isolated by flow cytometry sorting of (1) human breast cancer cell line MDA-MB-231, (2) primary cells from invasive breast cancer lesions and (3) primary cells from benign breast tissues. Real-time PCR is performed to determine the expression of stem cell genes including genes in Notch pathway. IHC is performed to determine the ER and PR status of the cells. Stem like and non-stem like cells are treated with GSI (a Notch inhibitor) and tamoxifen. The effects of the treatments on cell proliferation and apoptosis are determined by BrdU and Tunnel Assays.

Results and Conclusion: Previously we have shown that GSI alone effectively induced apoptosis in ER- MDA-MB-231 cells. Tamoxifen alone had substantial killing effects on ER+ MCF7 cells, but enhanced killing in both cell types were observed when treatments were combined. Stem like, non stem-like and unsorted cells were treated with GSI, tamoxifen and combination of GSI and tamoxifen. Proliferation was determined at 24 and 72 hours of treatment by BrdU assay. Stem-like cells exhibited significant sensitivity to GSI killing In addition, an enhanced effect was observed when GSI was combined with tamoxifen, suggesting that chemotherapy that targets both Notch signaling and estrogen receptor pathways in breast cancer stem cells may be an effective strategy in treating breast cancer.

Supporting data

See Figures 1-14.

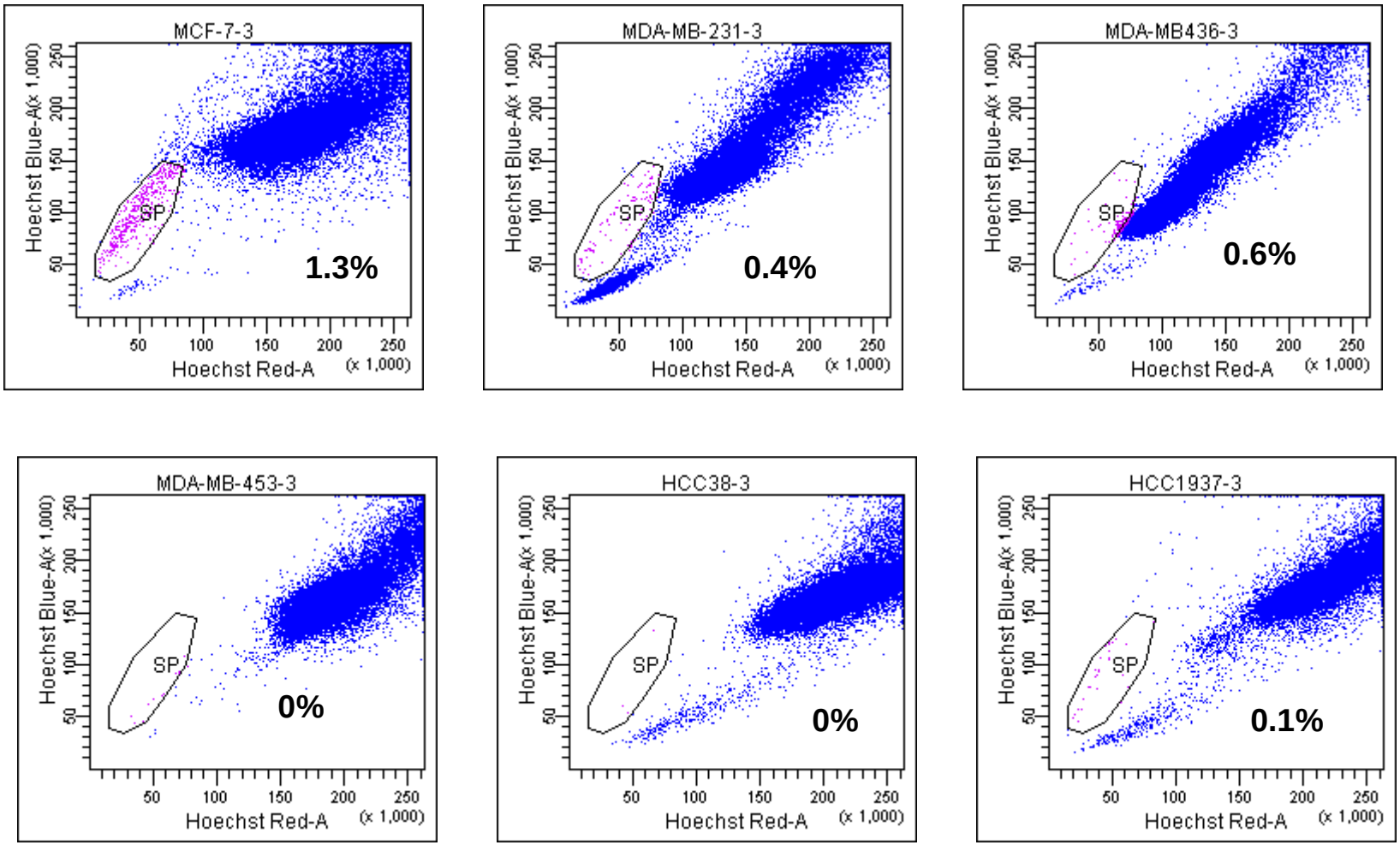


Figure 1. The Hoechst side populations in breast cancer cell lines. 10^6cells were loaded with 5μg/ml Hoechst 33342 for 90 minutes at 37°. Hoechst efflux was examined by BD LSR-II flow cytometer with UV laser at 360 nM for excitation and 405/20 BP filter for Hoechst blue and 675 LP filter for Hoechst red. The control cells were treated with 50mM Veripamil at the time of staining. The percentage of the side population is given for each panel.

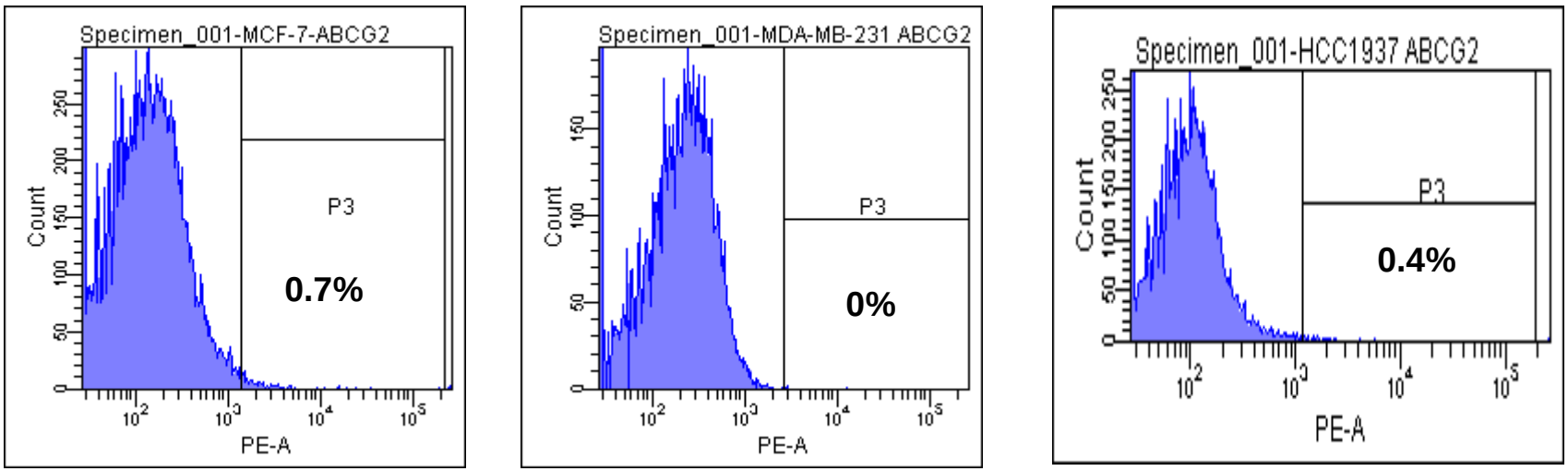
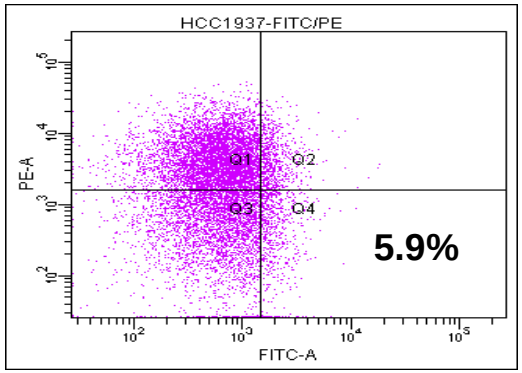
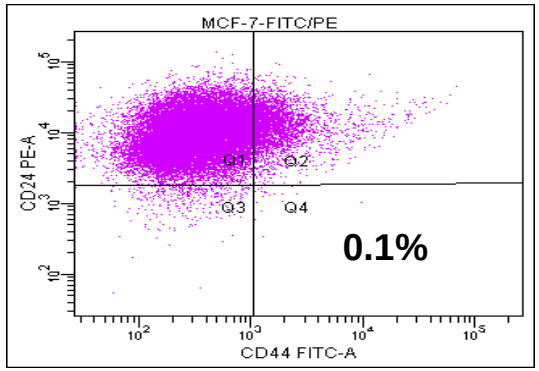
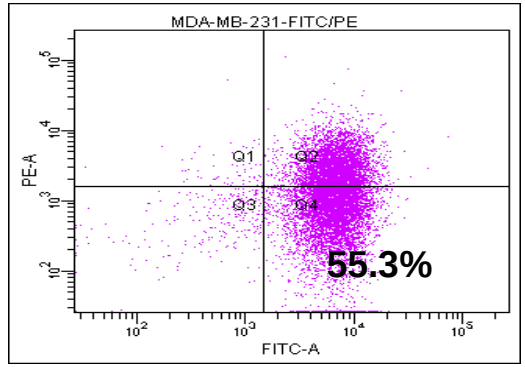


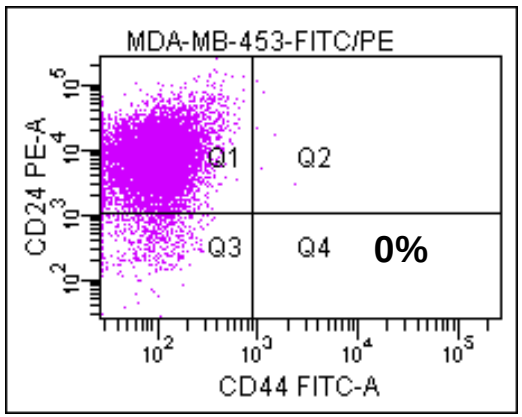
Figure 2 The flow cytometric analysis of ABCG2 expression in MCF-7, MDA-MB-23, and HCC1937 cells. 10^6 cells were stained with PE-conjugated anti-ABCG2 antibody (R&D Systems) according to the manufacturer's recommended protocol. ABCG2 expressing cells were determined against control cells stained with PE-conjugated isotype IgG and are expressed as percentage of total cells.

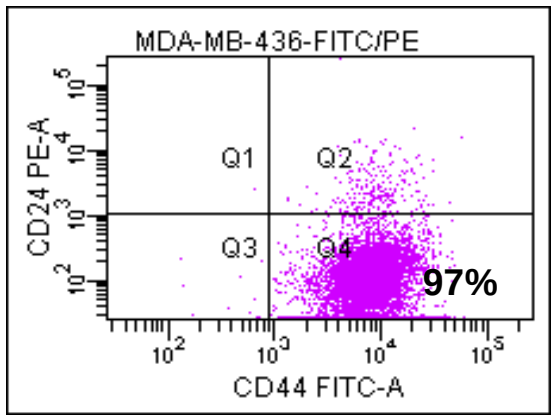
A

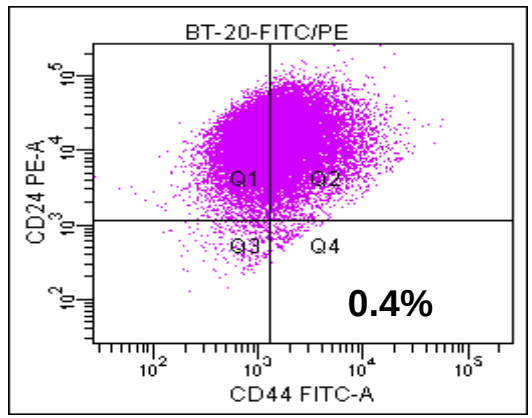

HCC1937-FITC/PE
PE-A
FITC-A
Q1
Q2
Q3
Q4
5.9%

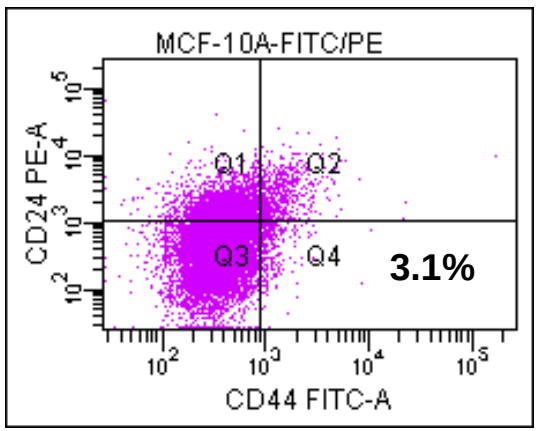

MCF-7-FITC/PE
CD24 PE-A
CD44 FITC-A
Q1
Q2
Q3
Q4
0.1%

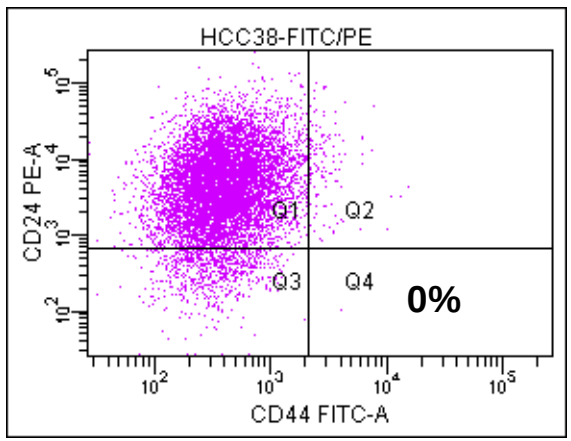

MDA-MB-231-FITC/PE
PE-A
FITC-A
Q1
Q2
Q3
Q4
55.3%


MDA-MB-453-FITC/PE
CD24 PE-A
CD44 FITC-A
Q1
Q2
Q3
Q4
0%


MDA-MB-436-FITC/PE
CD24 PE-A
CD44 FITC-A
Q1
Q2
Q3
Q4
97%


BT-20-FITC/PE
CD24 PE-A
CD44 FITC-A
Q1
Q2
Q3
Q4
0.4%


MCF-10A-FITC/PE
CD24 PE-A
CD44 FITC-A
Q1
Q2
Q3
Q4
3.1%


HCC38-FITC/PE
CD24 PE-A
CD44 FITC-A
Q1
Q2
Q3
Q4
0%

B

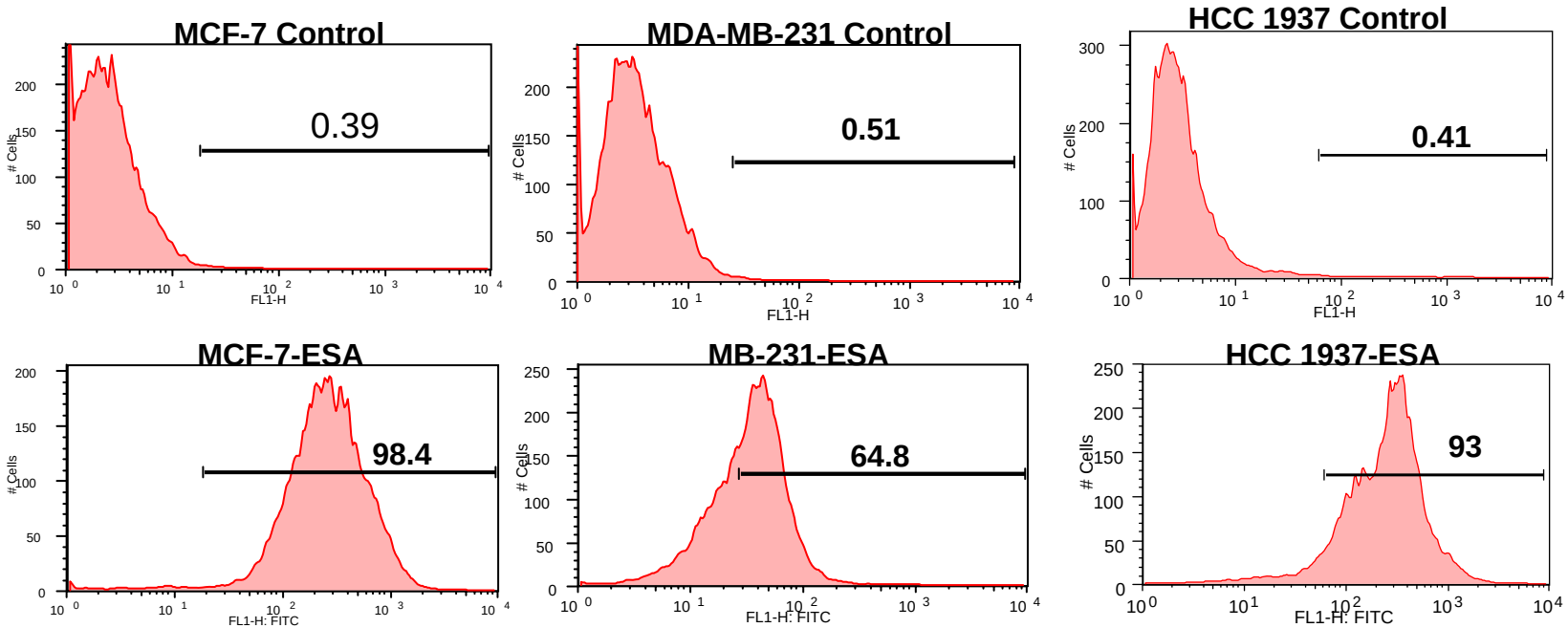


Figure 3. Flow cytometric analysis of CD44, CD24, and epithelial specific antigen (ESA)
A. Cells were stained with FITC-conjugated CD44 and PE-conjugated CD24 (BD Biosciences) according the protocol recommended by the manufacturer. Cells with $CD44^{+}/CD24^{-}$ phenotype were determined against cells stained with FITC or PE-conjugated IgG isotypes. Percentage of cells with $CD44^{+}/CD24^{-}$ is presented in each panel. **B.** Cells were stained with FITC-conjugated ESA (eBiosciences) and ESA positive cells (bottom panel) were determined against control cells stained with IgG isotype (top panels). The number in each panel represents the percent ESA positive cells.

.

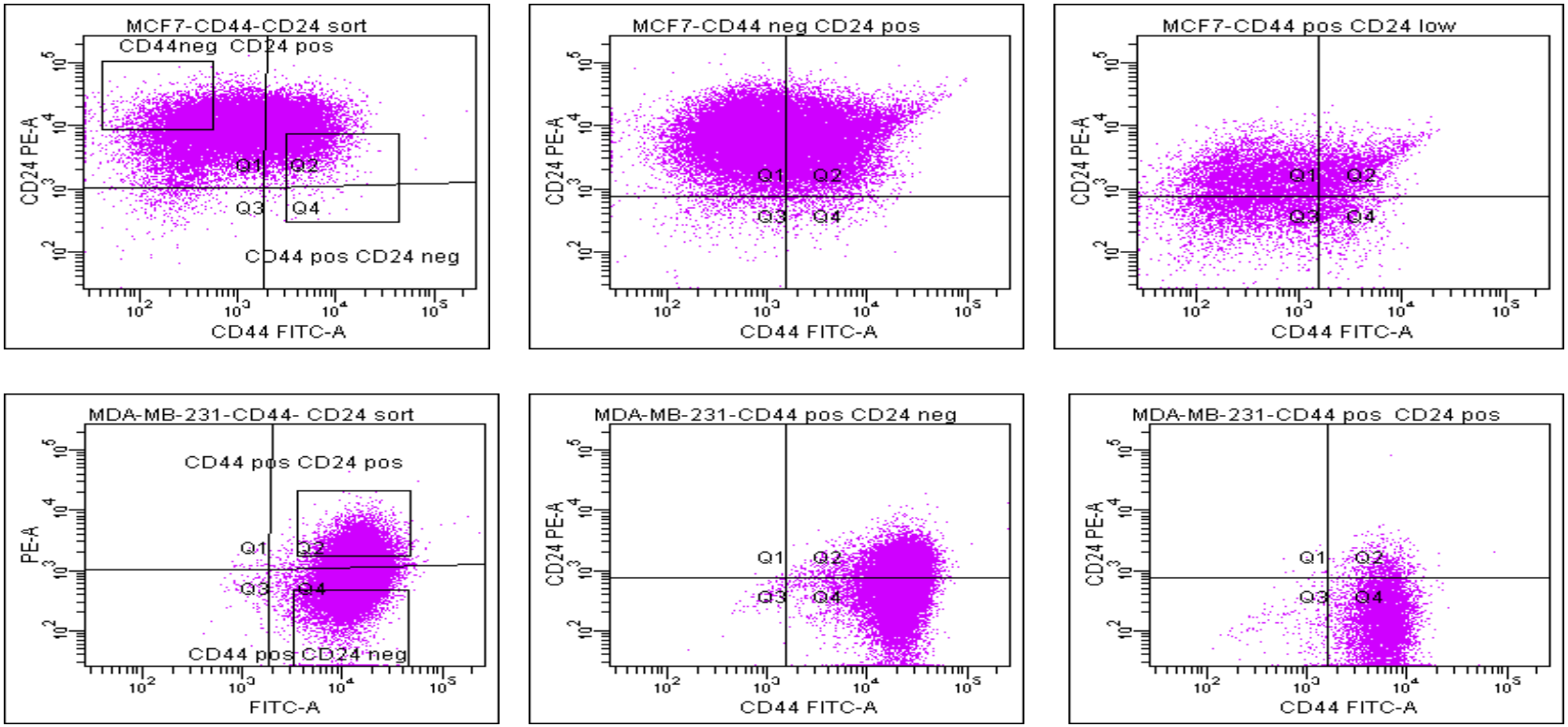


Figure 4. The switch of CD44/CD24 expression in presorted cells.
MCF-7 (Top panel) and MDA-MB-231 (bottom panel) cells were sorted by the surface markers CD44 and CD24 as shown in left column and then were cultured. After 2 weeks the presorted cells were re-examined for expression of CD44 and CD24 with the analysis of the original $CD44^{+}/CD24^{low}$ cells in the middle column and the analysis of the original $CD44^{-}/CD24^{high}$ cells in the right column.

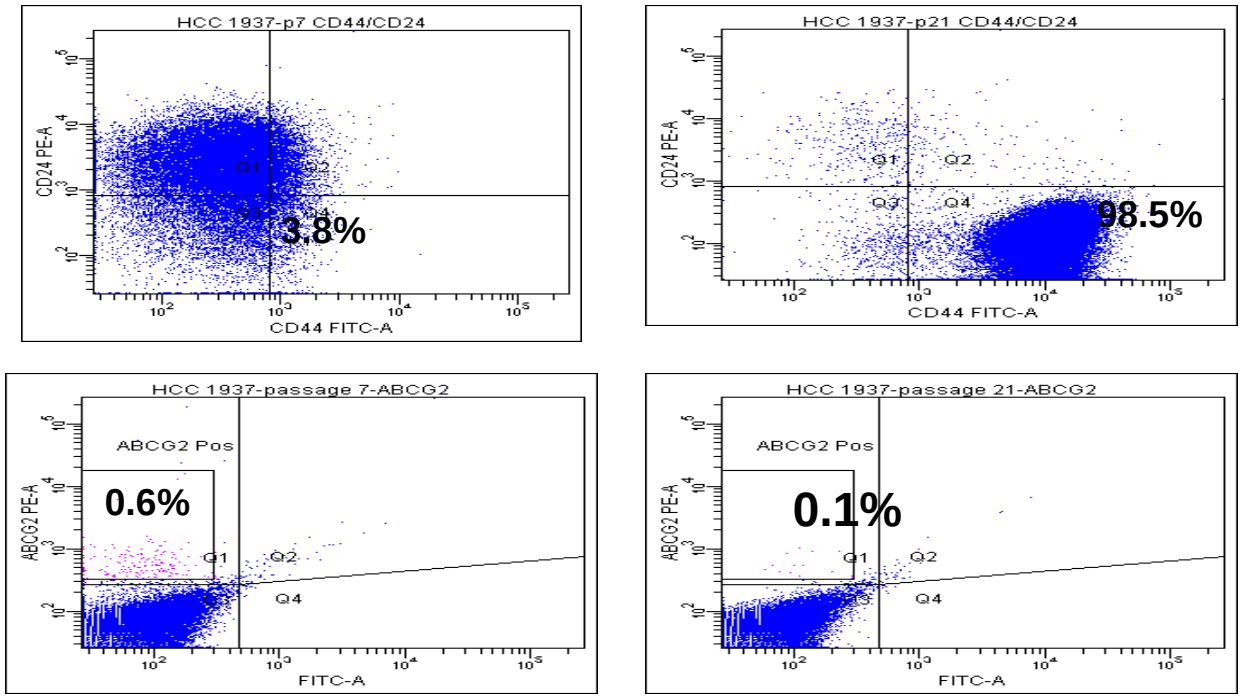


Figure 5. Altered expression of CD44/CD24 and ABCG2 surface markers with early and late passage of the HCC1937 cell line. Early (passage 7) and late (passage 21) HCC1937 cells were stained with CD44/CD24 (top panels) or ABCG2 (bottom panels). The percentage of cells with $CD44^{+}/CD24^{-}$ phenotype or positive ABCG2 were determined by flow cytometric analysis.

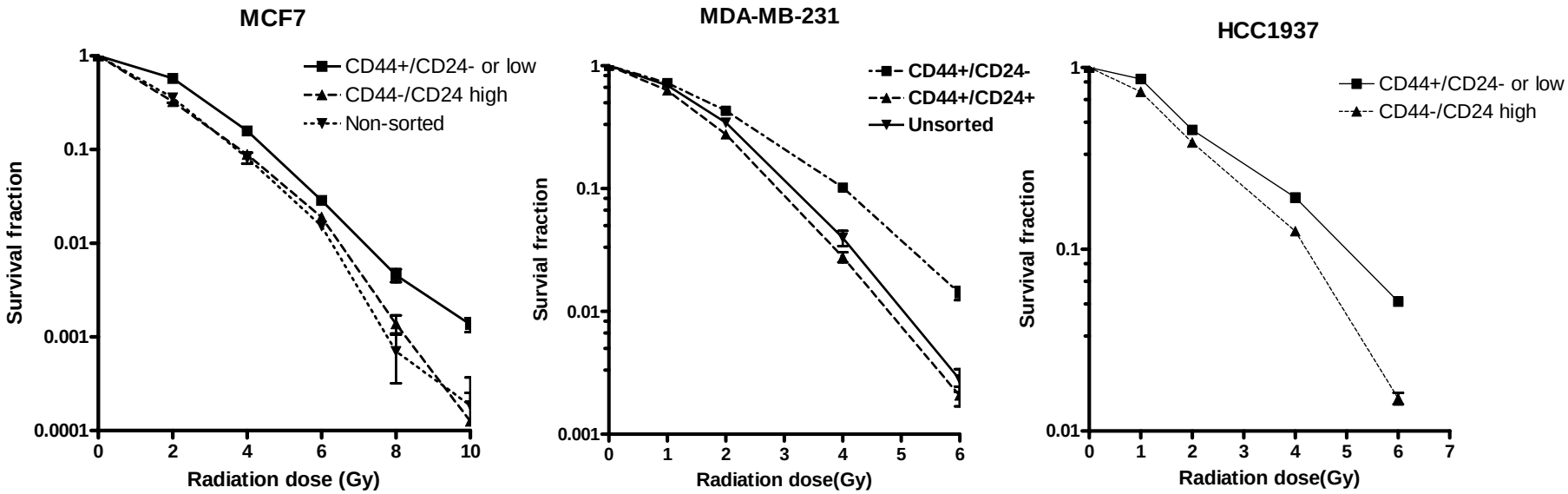


Figure 6. Post-radiation clonogenic survival analysis of CD44+/CD24- or low subpopulation vs. CD44-/CD24high (MCF-7 and HCC1937)and CD44+/CD24+(MDA-MB-231) subpopulations.

Cells were radiated 5 days after sorting and clonogenic survival analysis was conducted. On day 14 after radiation the colonies were counted and survival fraction was calculated as colonies formed divided by cell number seeded and corrected for plating efficiency

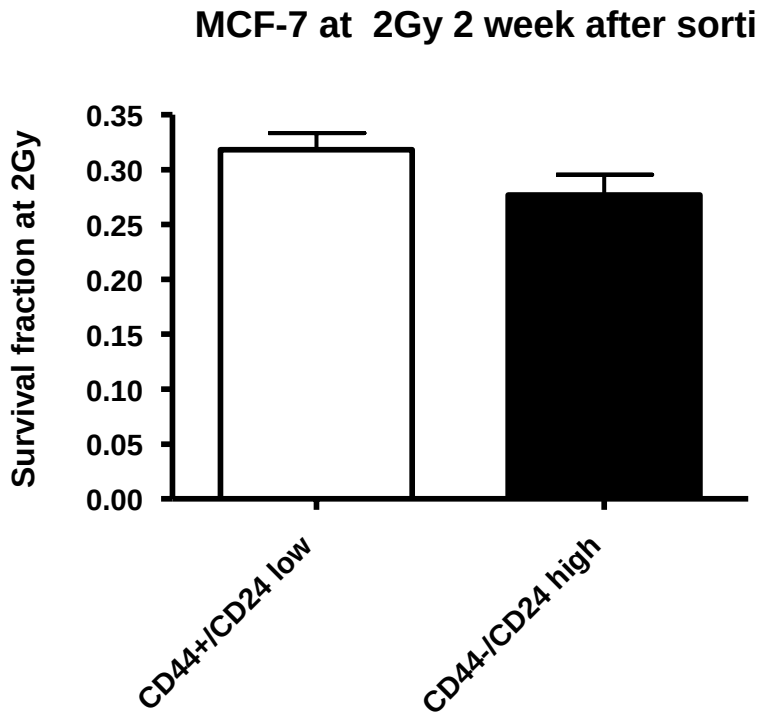


Figure 7 Post-radiation clonogenic survival analysis of MCF-7 subpopulations 2 weeks after sorting.

MCF-7 cells were sorted by CD44 and CD24 as $CD44^{+}/CD24^{low}$ and $CD44^{-}/CD24^{high}$ subpopulations After 2 weeks of culture, cells were exposed to radiation at 2 Gy. Clonogenic survival analysis was conducted and survival fraction at 2 Gy was calculated as described in Figure 6. Each column represents mean of three independent experiments $\pm$ SD.

Notch -1 expression

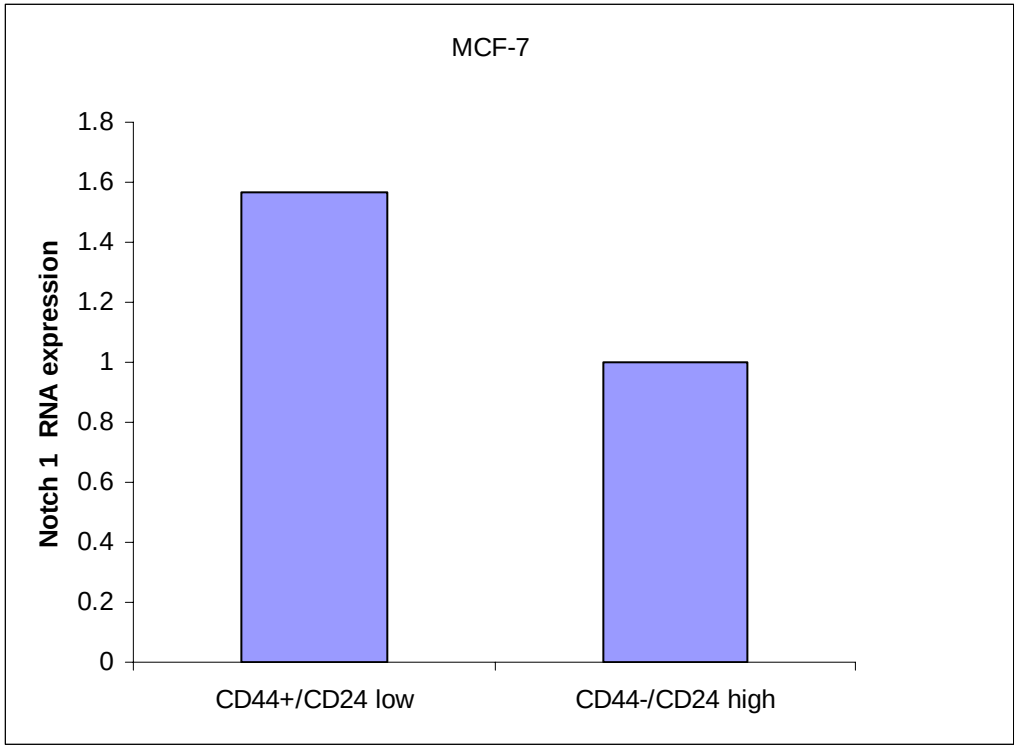


Figure 8. Notch 1 expression in $CD44^{+}/CD24^{low}$ and $CD44^{-}/CD24^{high}$ subpopulations of MCF-7 cells. MCF-7 cells were sorted according to the expression of CD44 and CD24. After 2 days of culture of the sorted cells RNA was extracted and real time RT-PCR was conducted. The relative expression of Notch1 was calculated by Δct of Notch1 over Δct of beta actin.

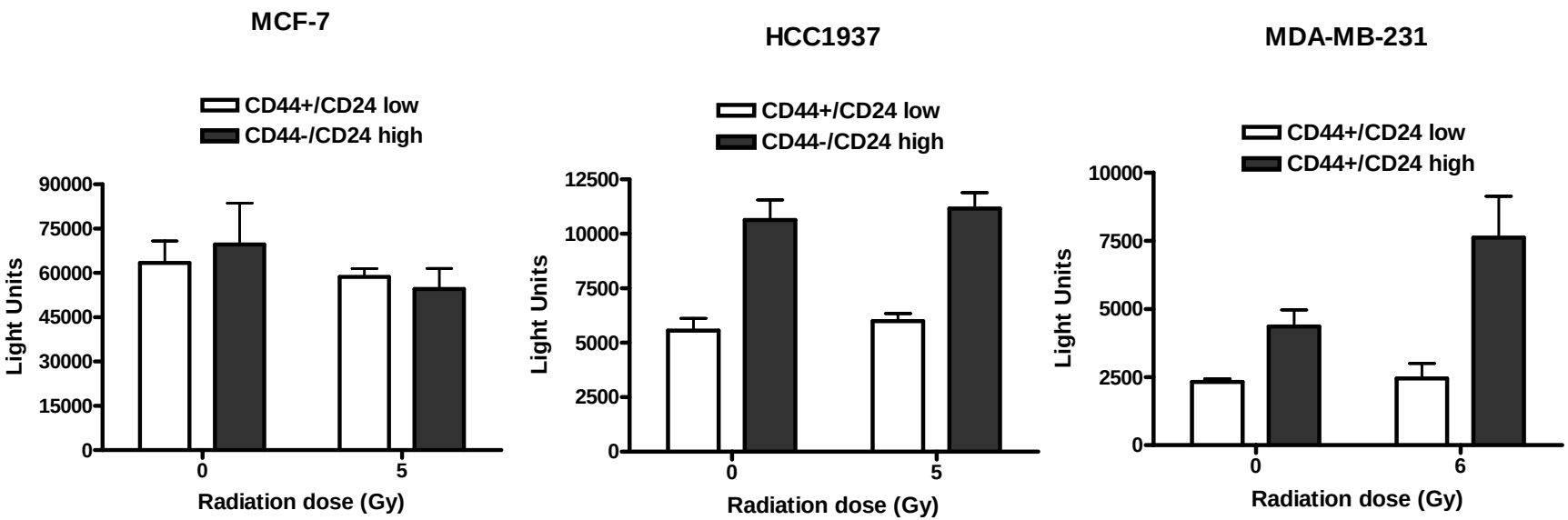


Figure 9. Analysis of In vivo ligation analysis activity among subpopulations of MCF-7, HCC1937, and MDA-MB-231 cell lines.

The pGL2 plasmid with SV-40 promoter driving the luciferase reporter gene, pGL2-Control, was linearized by Stu1 to create blunt ends between the promoter and luciferase reporter gene. The linearized plasmid was transfected into subpopulations of MCF-7, HCC193, and MDA-MB-231 cells prior and post radiation. The luciferase activity was measured 36 hours after transfection. The luciferase activity was standarized by β-gal activity for transfection efficiency and is shown as relative light units.

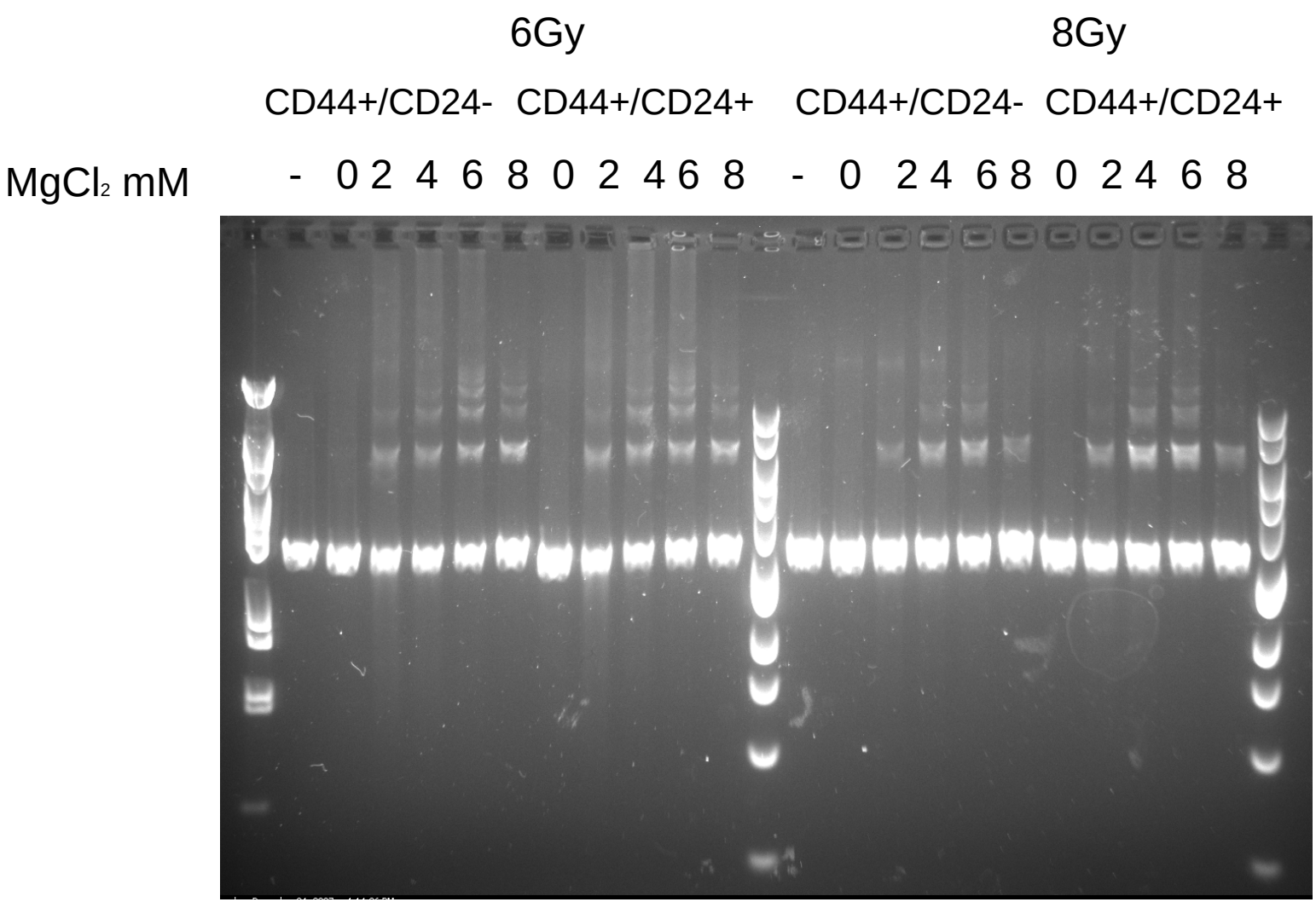
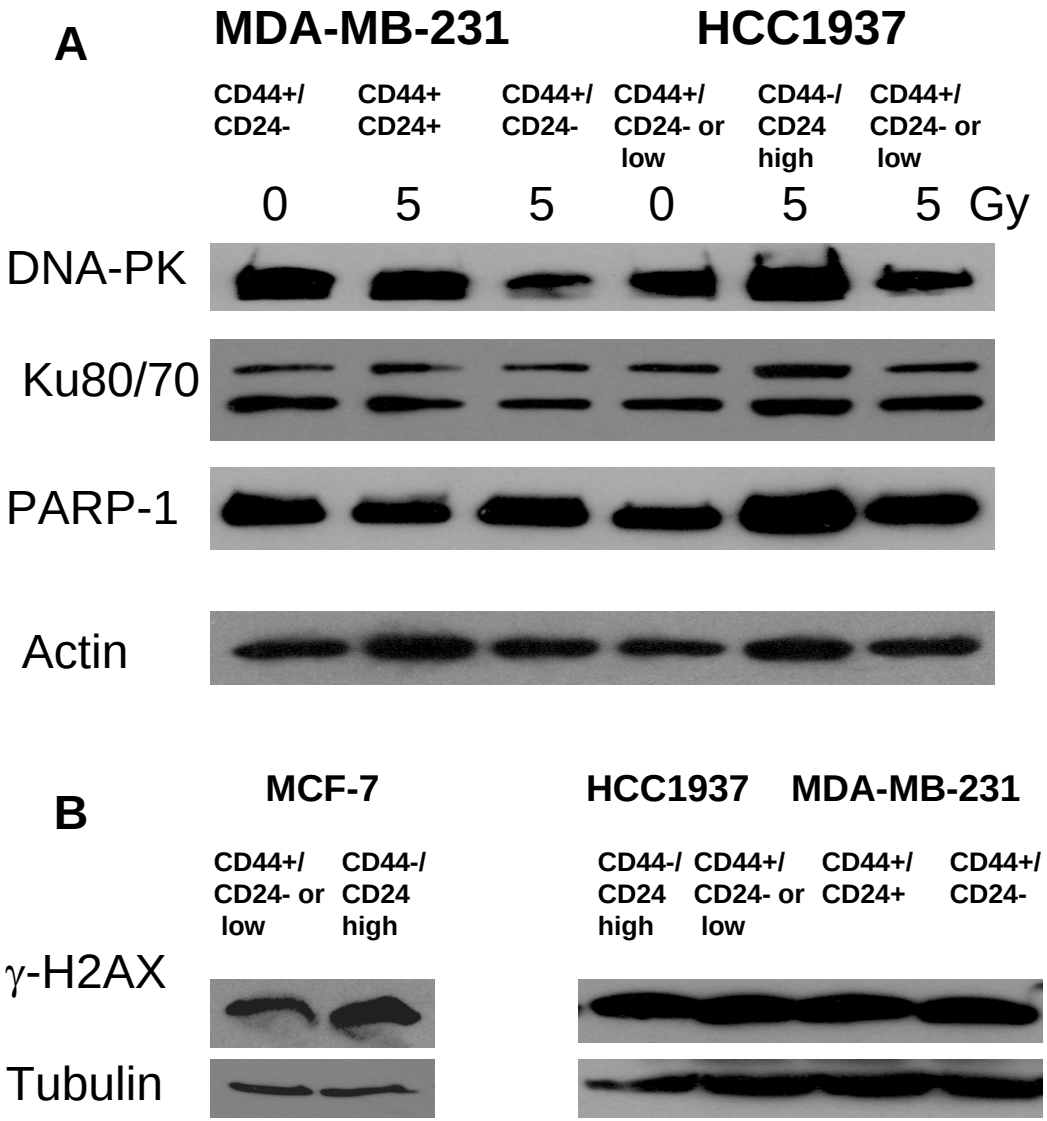
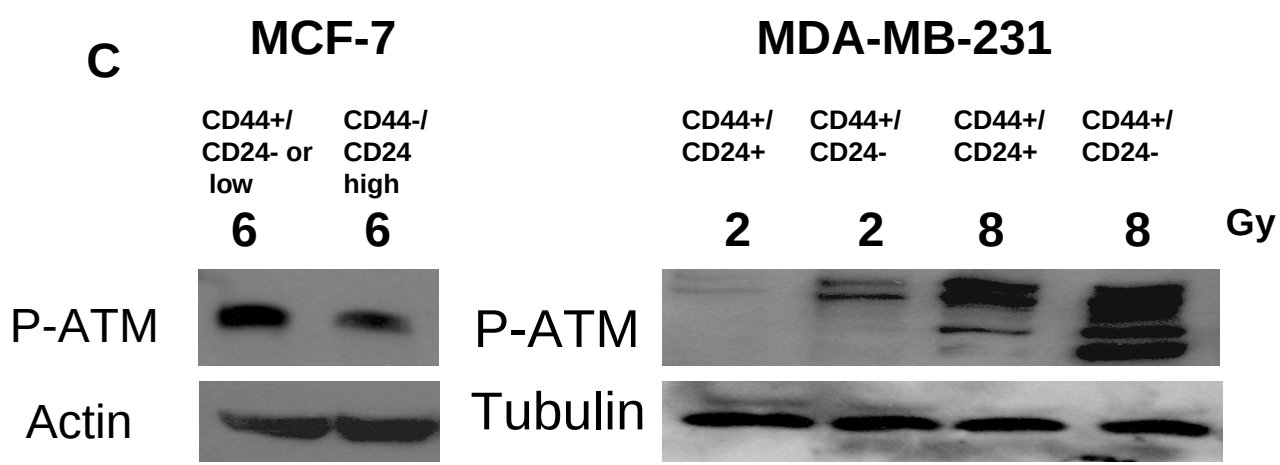
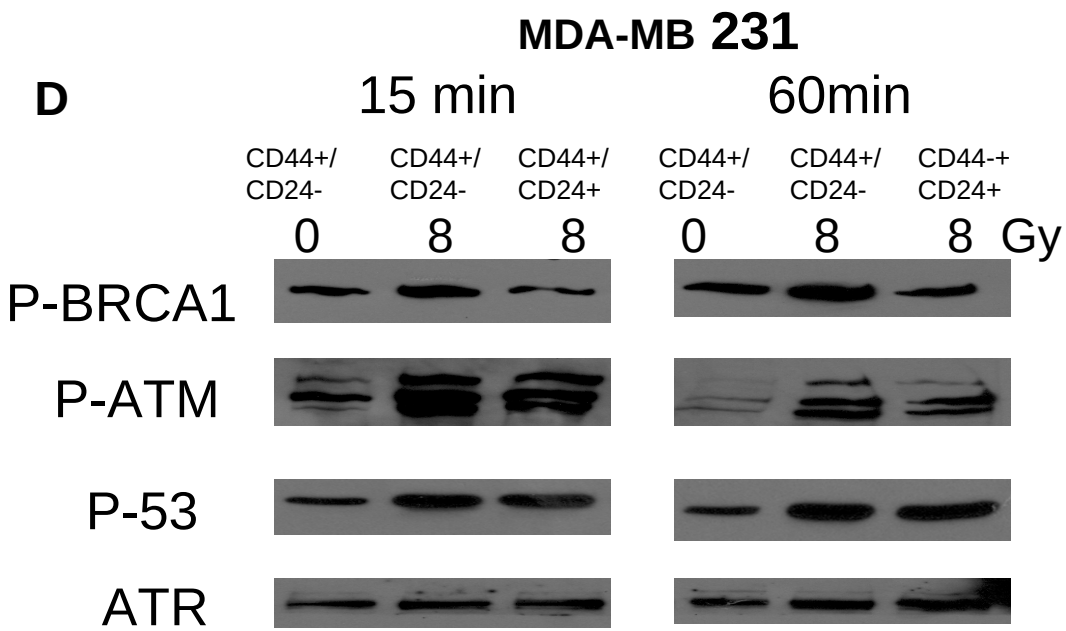


Figure 10. In vitro analysis of non-homologous end joining (NHEJ) activity in subpopulations of MDA-MB231 cells

Sorted MDA-MB231 cells were radiated and nuclear protein was extracted.
The analysis of NHEJ activity was conducted with plasmid DNA pBlue ks linearized with BamH1 and Xho1 in the absence or presence of varying concentrations of MgCl2.


A
MDA-MB-231
HCC1937
CD44+/ CD24-
CD44+ CD24+
CD44+/ CD24-
CD44+/ CD24- or low
CD44-/ CD24 high
CD44+/ CD24- or low
0 5 5 0 5 5 Gy
DNA-PK
Ku80/70
PARP-1
Actin
B
MCF-7
HCC1937
MDA-MB-231
CD44+/ CD24- or low
CD44-/ CD24 high
CD44-/ CD24 high
CD44+/ CD24- or low
CD44+/ CD24+
CD44+/ CD24-
γ-H2AX
Tubulin


C
MCF-7
MDA-MB-231
CD44+/ CD24- or low
CD44-/ CD24 high
CD44+/ CD24+
CD44+/ CD24-
CD44+/ CD24+
CD44+/ CD24-
6
6
2
2
8
8
Gy
P-ATM
P-ATM
Actin
Tubulin


MDA-MB 231
D
15 min
60min
CD44+/ CD24-
CD44+/ CD24-
CD44+/ CD24+
CD44+/ CD24-
CD44+/ CD24-
CD44-+ CD24+
0
8
8
0
8
8
Gy
P-BRCA1
P-ATM
P-53
ATR

E

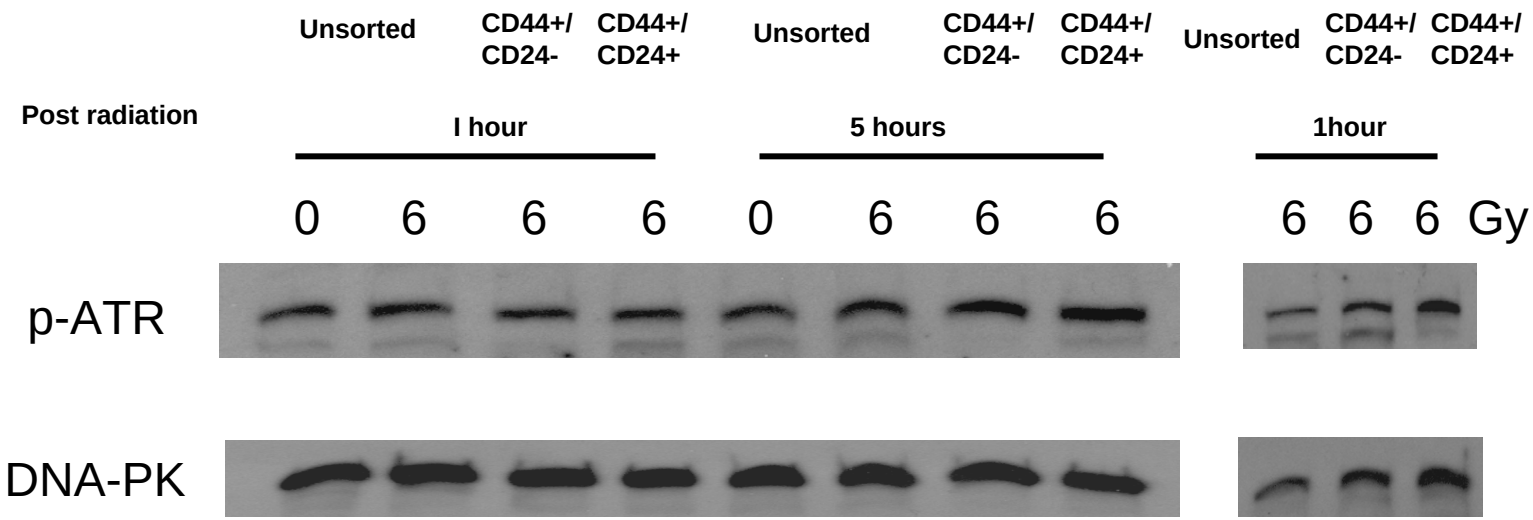


Figure 11. DNA damage-repair response after radiation in CD44+/CD24–or low vs. CD44-/CD24 high (MCF-7 and HCC1937) or CD44+/CD24+ (MDA-MB231) subpopulations. Western blot analysis of components of NHEJ (A) and phosphorylated H2AX(B), ATM(C and D), and ATR(E). Cells were sorted by FITC-conjugated CD44 and PE-conjugated CD24 and continued in culture for 5 days. The whole cell extract (MCF-7) or nuclear extract (MDA-MB-231 and HCC1937) was prepared one hour after radiation. Western analyses was conducted to detect the expression of components of NHEJ (A) and phosphorylated H2AX(B), ATM (C and D), and ATR (E).

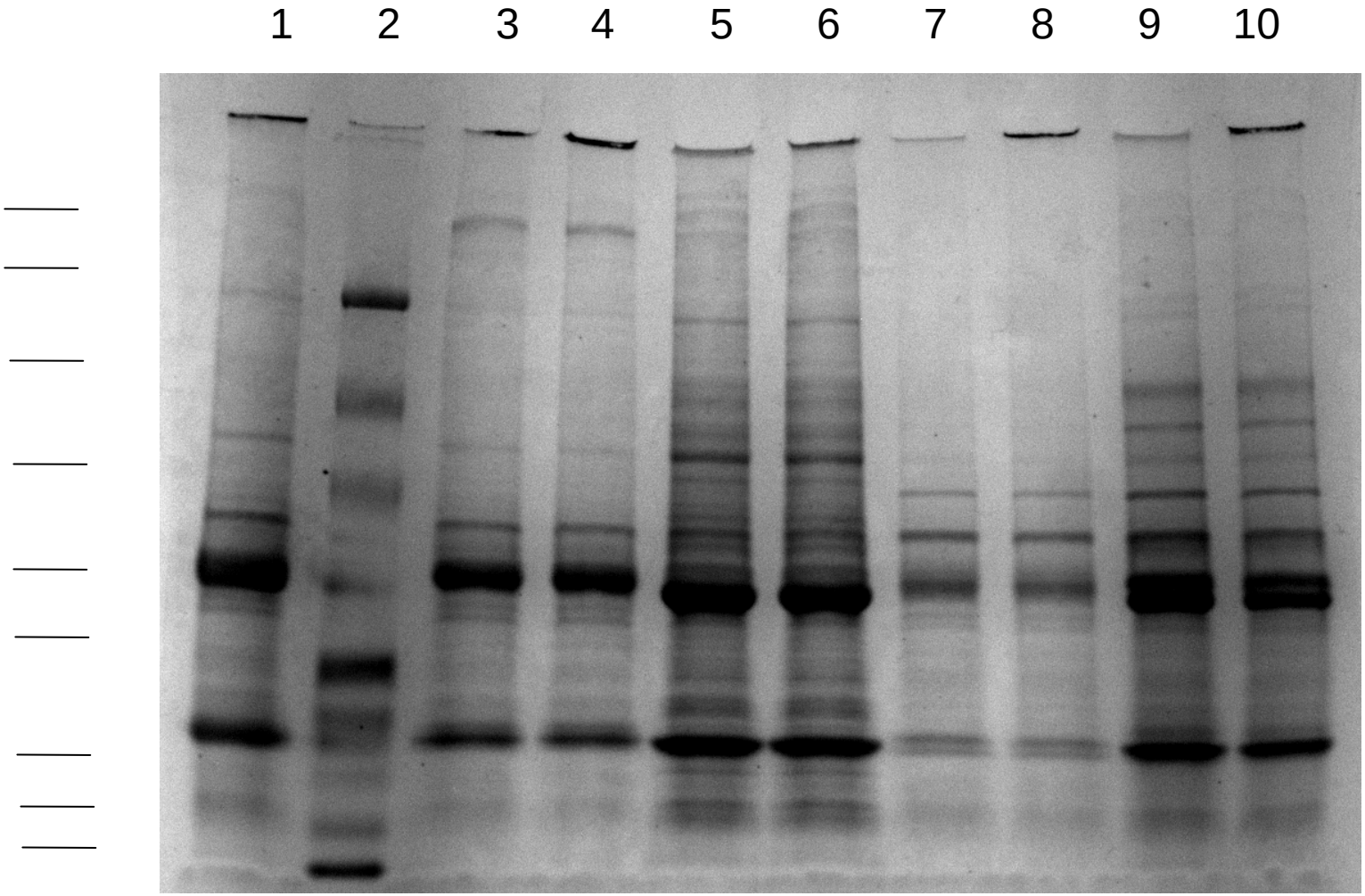


Figure 12 Illustration of co-immunoprecipitation ofnuclear proteins. Nuclear protein was extracted from MDA-MB 231 cells one hour after radiation at 10 Gy. Immunoprecipitation was conducted with antibodies against DNA-PKcs (lane 3 and 4), phosphorylated ATM (lane 5 and 6), Ku70 (lane 7 and 8), and Ku80 (lane 9 and 10). Lane 1, Control IgG only. Lane 2, molecular marker. Lane 1,3,5,7,and 9, nuclear extract from CD44+/CD24- cells and lane 4,6,8, and 10, nuclear extract from CD44+/CD24+ cells.

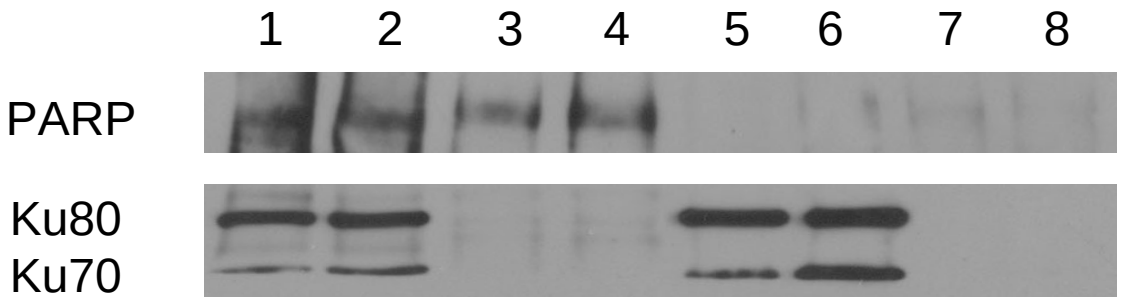


Figure 13. Phosphorylated ATM was correlated with PARP and Ku70/80 proteins. Nuclear protein was extracted from MDA-MB-231 cells one hour after radiation. Co-immunoprecipitation was conducted with antibody against phosphorylated ATM (lane 1and 2), PARP (lane 3 and 4), and Ku 80 (lane 5 and 6). Lane 7 and 8, co-precipitation by IgG. Co-precipitated PARP , Ku70 and Ku 80 were detected by antibody to PARP , Ku70, and Ku80 on the western blot .

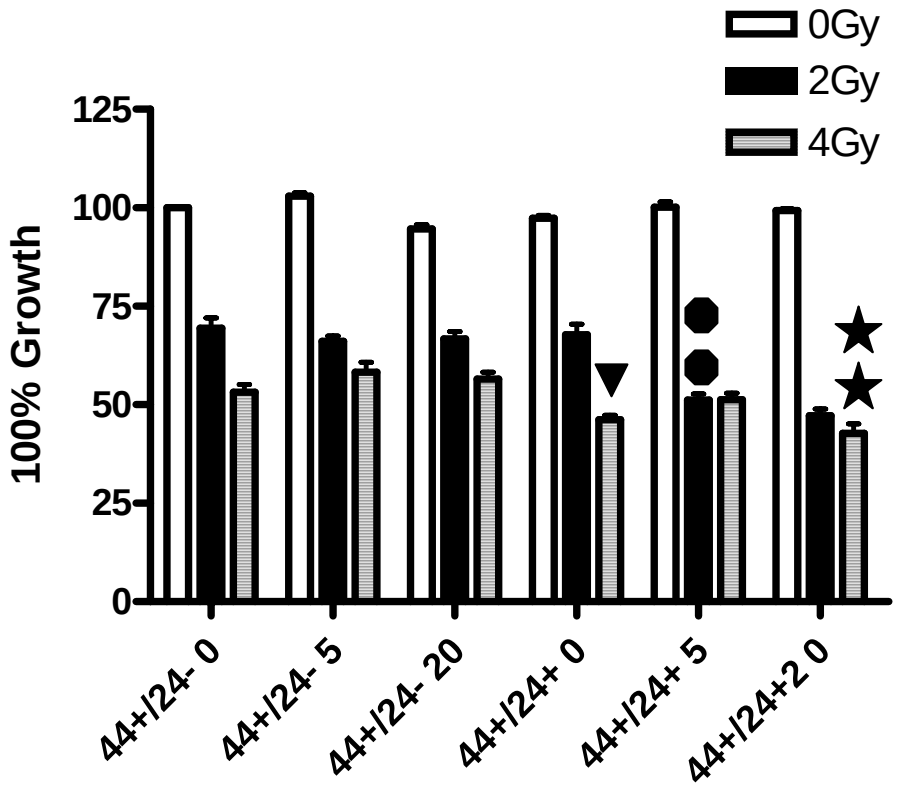


Figure 14. Proliferation assay of radiated MDA-MB231 cells treated with ATM inhibitor at 5 and 20 nM. Cells 6 days after sorting were treated with ATM inhibitor 2 hours before the radiation. 5000 cells were seeded in each well of a 96-well plate with the ATM inhibitor after radiation and fresh medium without ATM inhibitor was replaced at 24 hours. Proliferation assay was conducted with Promega's CellTiter96® AQueous One Solution Cell Proliferation Assay Kit after 7 days of culture. Solid triangle, $p<0.05$ between 44+/24+ 0 and 44+/24- 0. Double solid circle, $p<0.01$ between 44+/24+ 5 and 44+/24+ 0. Double solid star, $p<0.01$ between 44+/24+ 20 and 44+/24- 20. 0, 5, and 20 refer to the concentration of the ATM inhibitor.